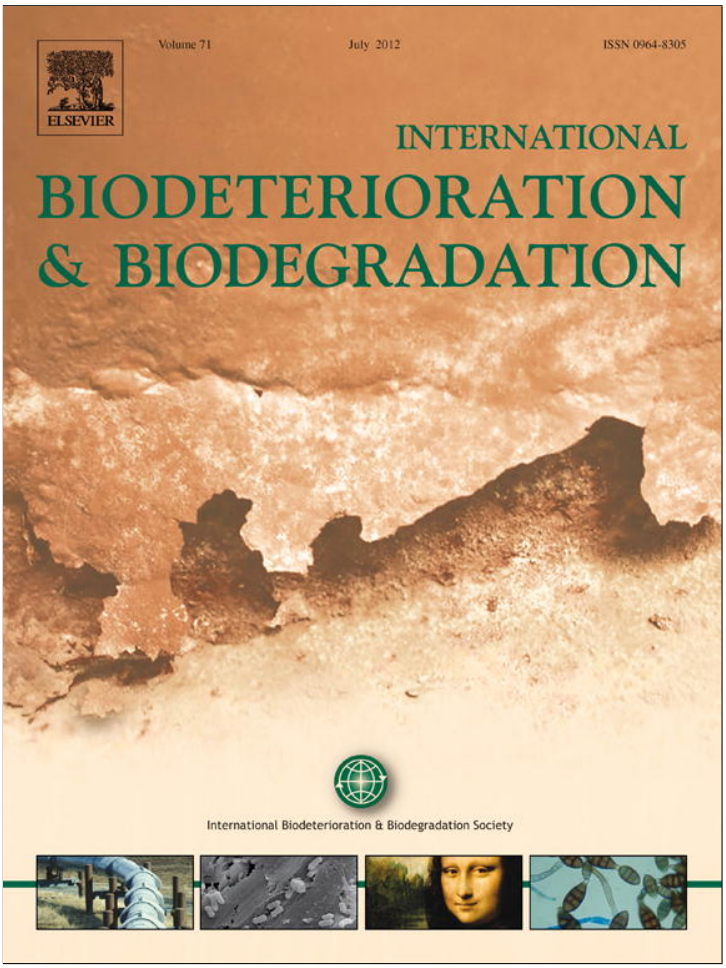

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Cr(VI) reduction by cell-free extracts of *Pichia jadinii* and *Pichia anomala* isolated from textile-dye factory effluents

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ABSTRACT

Chromium-resistant yeasts isolated from contaminated environments can be used to reduce toxic Cr(VI). This study assessed *in vitro* reduction of hexavalent chromium using crude chromate reductase (CChR) of *Pichia jadinii* M9 and *Pichia anomala* M10, two yeasts isolated from a textile-dye factory effluent. CChRs were characterized based on optimal temperature, pH, use of electron donors, metal ions and initial Cr(VI) concentration in the reaction mixture. Both CChRs showed an increase in Cr(VI) reductase activity with addition of NAD(P)H as electron donor and were highly inhibited by Hg^{2+} and Mn^{2+}. The CChR from *P. jadinii* M9 showed the highest chromate reductase activity at 60 °C and pH 6.0 in the presence of Cu^{2+} or Na^{+}, while *P. anomala* M10 CChR had the maximum activity at 50 °C and pH 7.0 in presence of Cu^{2+}. Initial Cr(VI) concentrations of 1.3 and 1.7 mM for CChRs of *P. jadinii* M9 and *P. anomala* M10 respectively were inhibitory.

1. Introduction

Chromium compounds are used extensively in numerous industrial processes, such as leather-tanning, metal plating and finishing, wood treatment, corrosion inhibition in power plants and nuclear facilities, and in the manufacturing of refractory materials, pigments, dyes, textiles and mining equipment, amongst others (Juvera-Espinosa et al., 2006).

The valence states of chromium (Cr) ranges from −2 to +6, but only the trivalent and the hexavalent forms appear to be of significance and are stable in the environment. Trivalent chromium is an essential trace element for all living organisms; it is necessary for fat and glucose metabolism and proper functioning of insulin. Cr(VI) compounds (CrO_4^{2-}, CrO_7^{2-}) are comparatively more toxic than Cr(III) due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids (Anderson, 1997). Accordingly, chromium and its compounds are placed on the priority list of toxic chemicals by USEPA (Thacker et al., 2007).

For this reason, it is important to develop an innovative, low cost, and eco-friendly method for the toxic heavy metal removal from the wastewater, instead of the conventional physical-chemical ones (Tseng and Bielefeldt, 2002; Juvera-Espinosa et al., 2006). Some microorganisms have developed the capabilities to protect themselves from heavy metal toxicity by various mechanisms such as adsorption, uptake, methylation, oxidation, and reduction (Ramírez-Díaz et al., 2008).

Cr(VI) enters the cell through non-specific sulfate transporters by facilitated diffusion (Wiegand et al., 1985). The Cr(VI) gradient between the two sides of the cell membrane is maintained by the metabolically active cells themselves, which continuously reduce the accumulated Cr(VI) to lower oxidation states by both enzymatic processes (involving flavoenzymes) and non-enzymatic processes (glutathione (GSH), NADPH and ascorbate) (Pesti et al., 2000). A wide variety of bacteria have been reported to reduce Cr(VI) to Cr(III) under aerobic and/or anaerobic conditions (Camargo et al., 2004; Bae et al., 2005; Elangovan et al., 2006; Polti et al., 2009; Kanmani et al., 2011). The enzymatic reduction of Cr(VI) involves a soluble cytosolic chromate reductase under aerobic conditions or a membrane-bound chromate reductase during anaerobic respiration, where chromate acts as the electron final acceptor (Cheung and Gu, 2007). Although microbes of the genera *Pseudomonas*, *Arthrobacter*, *Escherichia* and *Bacillus* have been reported to reduce Cr(VI) through soluble chromate reductase, only a few of these

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enzymes have been characterized (Desai et al., 2008; Elangovan et al., 2010; Liu et al., 2010).

Yeasts are known to play an important role in the removal of toxic heavy metals (Ksheminska et al., 2005; Guillén-Jiménez et al., 2008; Villegas et al., 2008). Furthermore, the occurrence of indigenous Cr(VI)-reducing eukaryotic microorganisms, including those with no history of Cr(VI) contamination, has provided important non-conventional yeasts with significant biological relevance and biotechnological applications. Some authors argue that the microbial reduction of Cr(VI) can be considered as an additional mechanism of resistance to chromate, which is usually not encoded in plasmids (Cervantes et al., 2006). The enzymatic biospeciation of Cr(VI) to Cr(III) with eukaryotic microorganisms was reported in *Candida maltose* (Ramirez–Ramirez et al., 2004) and *C. utilis* (Muter et al., 2001) and fungi such as *Hypocrea tawa* (Morales-Barrera et al., 2008) and *Aspergillus* sp. (Srivastava and Thakur, 2006). However, no purification or characterization of the proteins involved were performed in these cases. In this context, the study of specific chromate reductases would be interesting in order to advance in the knowledge of the cellular mechanisms involved in bioremediation processes. The objective of this work was to describe the characterization of crude chromate reductase activity from yeast strains *Pichia jadinii* M9 and *Pichia anomala* M10, isolated from textile dye polluted sites, selected by its high Cr(VI) tolerance.

2. Materials and methods

2.1. Microorganisms and culture conditions

Chromate-resistant yeasts *P. jadinii* M9 and *P. anomala* M10, previously isolated from textile-dye factory effluents (Tucumán, Argentina) were used (Fernández et al., 2010).

To obtain the biomass for the crude chromate reductase, the yeasts were grown in 200 ml of Modified Yeast Nitrogen Base Medium (MYNB) during 48 h supplemented with 1 mM Cr(VI) and without chromium (control). Yeast strains grown for 24 h in Czapeck malta medium (CMM) (Fernández et al., 2010), were used as inoculum (10% v/v). MYNB composition was Yeast Nitrogen Base (DIFCO-YNB w/o aminoacids and $(NH_4)_2SO_4$, 10 × −10%) supplemented with sucrose (50 g l^{-1}) and $(NH_4)_2SO_4$ (0.6 g l^{-1}), pH 5.0. All the experimental sets were performed on a rotary shaker (250 rpm) at 25 °C according to previous experimentations (Fernández et al., 2009, 2010).

The Cr(VI) (as $K_2Cr_2O_7$ or K_2CrO_4) stock solution was prepared in double distilled water and filter-sterilized (0.2 μm-cellulose acetate membrane filter; SARTORIUS).

2.2. Crude chromate reductase preparation

The biomass obtained as above explained, was harvested by centrifugation at 10,000 × *g* for 10 min. Pellets were washed twice with 50 mM phosphate-citrate buffer (pH 5.0) and resuspended in the same buffer with 50 μl of a protease inhibitor cocktail (Set I CALBIOCHEM) plus a volume of sterilized glass beads. Cells were disrupted by sonication for 5 min in cold conditions (5 cycles: 59 seg on, 30 seg off; Sonics Vibra Cell VCX 130). The homogenate was centrifuged at 10,000 × *g* for 10 min at 4 °C to remove cell walls and unbroken cells. After filtering through a 0.2 μm-cellulose acetate membrane, the filtrate was used as cell-free extract (CFE). After that, CFE was fractioned with ammonium sulfate and the precipitate corresponding to the 70–90% (w/v) salt concentration fraction was redissolved in 50 mM phosphate-citrate buffer (pH 5.0). The fractioned sample was dialyzed against the same buffer overnight and was used as crude chromate reductase (CChR).

2.3. Chromate reductase assay and protein estimation

Decrease of chromate concentration by CChR was assayed at 30 °C using 50 μl of sample preparation in 0.25-ml reaction mixtures containing (to a final concentration): 50 mM phosphate-citrate buffer (pH 5.0), 0.5 mM K_2CrO_4, 1 mM NADH (Polti et al., 2009). The reaction was started by addition of chromate to the reaction mixture and the residual chromate concentration was measured at final point after 30 min. Controls without NADH or CChR were also assayed.

Cr(VI) in solution was determined by colorimetric measurement of the pink-violet colored complex formed after reaction with diphenyl carbazide (DPC) in acid solution (Urone, 1955). Total protein was determined with a bicinchoninic acid (BCA) kit (Sigma), using bovine serum albumin as standard.

2.4. Characterization of crude chromate reductase activity

Chromate reductase activity was measured at 30 °C and at different pH values using several buffers (50 mM phosphate-citrate, pH 4.0–5.0; 50 mM phosphate, pH 6.0–8.0 and 50 mM TRIS-HCl, pH 8–9). For pH stability, the CChRs were incubated in different buffers (pH 4.0–9.0) at 8 °C for 24 h and the residual activity was measured.

Effect of temperature was studied by measuring chromate reductase activity at different incubation temperatures between 10 and 100 °C at pH 5. For thermal stability, the CChRs were incubated between −20 °C and 73 °C at pH 5 for 24 h, cooled in an ice bath when appropiate, and the residual chromate reductase activity of all samples was measured.

To evaluate the saturating concentration of Cr(VI), several reaction mixtures with different Cr(VI) initial concentrations from 0.4 to 2.0 mM were tested. Samples from these reaction mixtures were taken at different times to determine the residual Cr(VI) concentration.

The CChRs samples were also treated with several metal ions to a final concentration of 1 mM at optimal pH and temperature for each CChR; Na^+, Ca^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+} were tested by using 10 mM solutions of Na_2SO_4, $CaCl_2$, $CuCl_2$, $HgCl_2$, $MgCl_2$, $MnSO_4$, $ZnSO_4$ and $FeCl_3$, respectively. The electron donors tested were NADH and NADPH in a final concentration of 1 mM. Controls without CChR were assayed in order to discard the unspecific Cr(VI) reduction by both cofactors.

Activity of the chromate reductase without pretreatment was considered 100%. The rate of reaction was expressed as the number of nmoles of Cr(VI) reduced in 1 min by 1 mg of total protein in the sample.

2.5. Statistical analysis

All values and data points presented in this work are the means of at least triplicate determinations of independent assays. Data were analyzed using the GraphPad InStat Instant Biostatistics package version 3.0. Statistical analysis was conducted using Minitab (Minitab Inc., State College, PA,USA).

3. Results and discussion

A variety of mechanisms exist for removal of heavy metals from aqueos solution by microrganisms and higher plants. The cellular response to the presence of metals include various processes such as biosortion by cell biomass, active cell transport, binding by cytosolic molecules, entrapment into cellular capsules,

precipitation and oxidation–reduction reactions, as well as protein-DNA adduct formation and induction of stress proteins (Zahoor and Rehman, 2009).

Strains *P. jadinii* M9 and *P. anomala* M10, isolated from a tannery effluent, were resistant to chromium (up to concentration of 2 mM) and showed Cr(VI) reduction activity. In culture medium, hexavalent chromium was reduced to undetectable levels within 96 and 72 h of incubation for *P. jadinii* M9 and *P. anomala* M10 respectively (Fernández et al., 2009, 2010). Experiments with cell free extracts of *P. jadinii* M9 and *P. anomala* M10, could indicate that a soluble type of enzymes were responsible for Cr(VI) reduction. Considering the ability of the strains under study, a protocol to obtain CChR was developed (sonication with glass beads and fractionated precipitation) and the subsequent characterization was evaluated. Up to now, there are no reports available on yeasts crude chromate reductase characterization.

As shown in Fig. 1(a), the activity of CChR obtained from cells grown in the absence or presence of 1 mM Cr(VI) was almost equal irrespective of the growth conditions. In the case of CChR from *P. jadinii* M9 a raise in the specific activity was observed with 1 mM Cr(VI), probably meaning an induction of the chromate reductase activity due to the presence of Cr(VI). Similarly, Das and Chandra (1990) studied a strain of *Streptomyces* sp. M3 and noticed an increase in the chromate reductase activity when working in cultures with Cr(VI). These same authors found that this enzyme expression was constitutive. There were also discovered other chromate reductase enzymes with constitutive expression in *Bacillus* species (Garbisu et al., 1998; Pal et al., 2005; Desai et al., 2007). In the case of constitutive expression, this activity might be not specific for this metal and, therefore, normally expressed in cells. It could also be the result of induction by some other components of the culture medium with or without Cr(VI). Kwak et al. (2003) reported the presence of chromate reductase activity in *Vibrio harveyi*, which also had nitroreductase activity. In *Pseudomonas denitrificans*, the iron reductase (Ferb) also had chromate reductase activity (Mazoch et al., 2004). Taking into account the total protein concentration, this was twice higher in the presence of the metal in the CChR of both yeasts strains (Fig. 1(b)). Thus, the real value of chromate reductase activity might be masked by the amount of proteins not related to this specific activity, but possibly to other protective mechanisms against the stress produced by this metal toxicity.

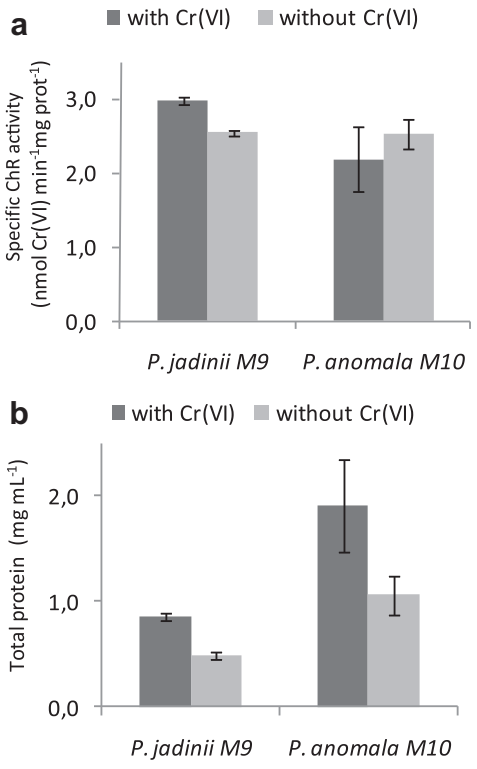


Fig. 1. Specific Chromate reductase activity (a) and total protein (b) content of *Pichia jadinii* M9 and *Pichia anomala* M10 CFEs obtained from cultures amended with and without Cr(VI). Data are mean of three independent assays and error bars indicate standard deviation.

Essentially, no chromate reductase activity was obtained in heat denatured CChR. Chromate reductase activity was negligible to almost nil in supernatant from both strains obtained after harvesting the cells, additionally, chromate reductase activity was found in entire cells resuspended in phosphate-citrate buffer. These results also reinforced the idea that chromate reductase activity was associated with the soluble fraction of the cells and was not extracellular (data not shown).

3.1. Characterization of crude chromate reductase activity

3.1.1. Temperature

The effect of temperature in the range 10–100 °C was evaluated. In the CChR of *P. jadinii* M9 a maximum at 60 °C was observed (Fig. 2), in the case of *P. anomala* M10 the higher chromate reductase activity was observed at 50 °C. Among bacterial CChRs, the optimal temperature varies in the range 30 °C–50 °C (Bae et al., 2005; Elangovan et al., 2006; Sarangi and Krishnan, 2007), as no CFEs or CChRs with chromate reductase activity had been described in yeasts yet, there is nothing to compare with. In both CChRs assayed, another maximum value was observed at an unusually high temperature, 99 °C; being difficult to relate this value with an enzymatic activity, moreover it could be due to a non-protein metabolite. Among the low molecular mass reductants, glutathione (GSH) is widespread in yeasts (Penninckx and Elsekens, 1993) and seems to be the most important agent participating in the non-enzymatic reduction of Cr(VI). Nevertheless, further studies are being conducted to elucidate this phenomenon.

Following incubation of the CChRs for different periods of time at various temperatures, their residual activity was measured at 30 °C (Fig. 3). For *P. jadinii* M9 CChR, incubation at 55 °C produced a reduction in activity of a 55%, after incubation at 73 °C the residual activity was reduced to 68% compared with the CChR kept

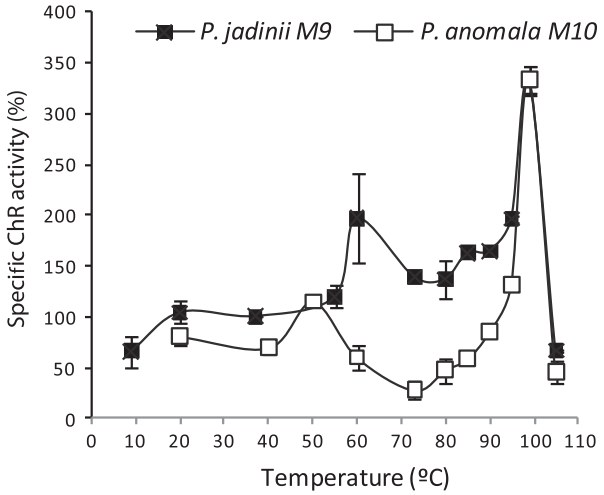


Fig. 2. Effect of incubation temperature in the Chromate reductase activity of CChRs from *Pichia jadinii* M9 and *Pichia anomala* M10. Data are mean of three independent experiments and error bars indicate standard deviation.

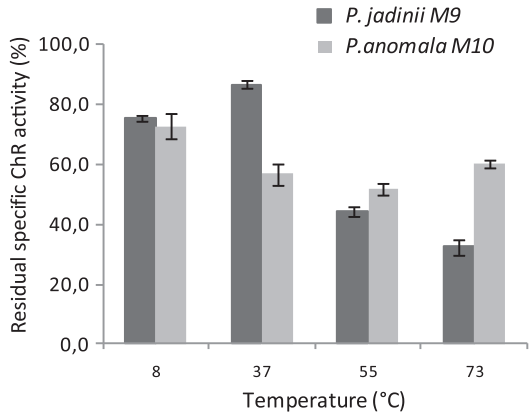


Fig. 3. Thermal stability of CChRs from *Pichia jadinii* M9 and *Pichia anomala* M10. Data are mean of three independent assays and error bars indicate standard deviation.

at −20 °C (control). In *P. anomala* M10 CChR when incubated at 8 °C a decrease in activity of 25% was observed and at 50 °C the activity was 50% compared with the control (−20 °C). For *Escherichia coli* and *Bacillus* sp. CFEs, thermal stability was up to 30 °C (Bae et al., 2005; Elangovan et al., 2006). On the contrary, *Pseudomonas putida* CFE probed to be more resistant, keeping its stability up to 50 °C (Park et al., 2000).

3.1.2. pH

To determine the optimum pH, the chromate reductase activity of the CChRs was measured at different pH values using several buffers (Fig. 4). In the CChR of *P. jadinii* M9, the higher activity was observed in phosphate-citrate buffer pH 6.0; nevertheless a marked loss of activity was observed when the pH changed to higher or lower values. The CChR of *P. anomala* M10 exhibited its higher activity in phosphate buffer pH 7.0. In this case there was a decrease in activity when increasing the pH value, however at pH values below the optimum, the decrease in activity was not as pronounced. When pH stability was tested, *P. jadini* M9 CChR probed to be stable in the range of 5.0–9.0, at pH 4 and 10 a lost in activity of 55% and 20% was observed (Fig. 5). On the contrary, in *P. anomala* M10 CChR, pH stability was moderately stable in the range of 4.0–6.0, nevertheless its optimum pH was 7.0. This may indicate more acidic pH stability. Other authors reported stability

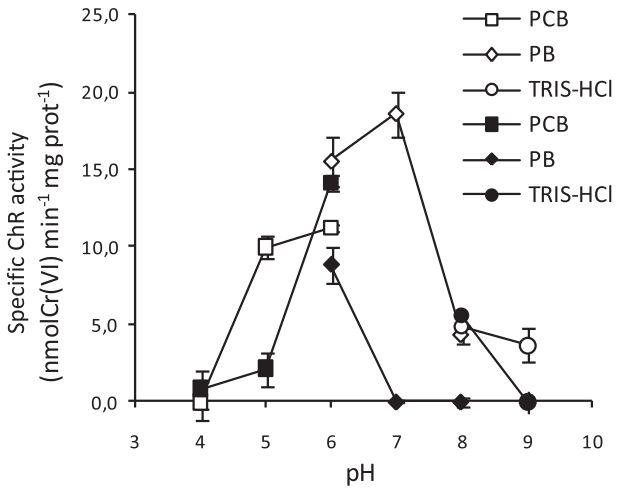


Fig. 4. Effect of pH and buffer composition in the Chromate reductase activity of CChRs from *Pichia jadinii* M9 (black markers) and *Pichia anomala* M10 (white markers). Data are mean of three independent experiments and error bars indicate standard deviation. CPB: citrate-phosphate buffer, PB: phosphate buffer, TRIS-HCl buffer.

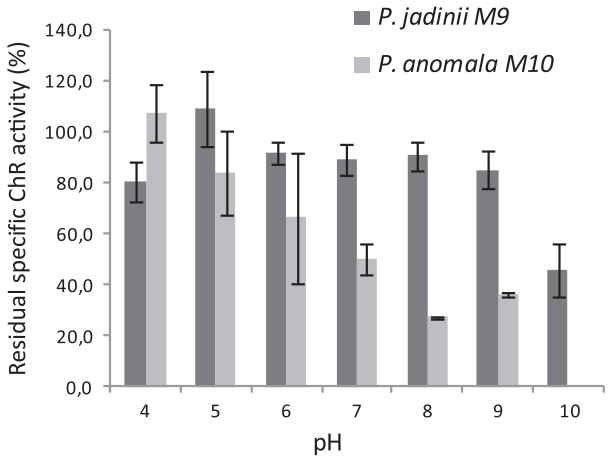


Fig. 5. pH stability of CChRs from *Pichia jadinii* M9 and *Pichia anomala* M10. Data are mean of three independent assays and error bars indicate standard deviation.

between 6.5 and 7.5 in *E. coli* CFE (Bae et al., 2005) and in the range of 5.0–8.0 in *Bacillus* sp. (Elangovan et al., 2006).

3.1.3. Electron donors and metal ions effects on chromate reductase activity

The effects of electron donors, inhibitors and metal ions on chromate reduction by CChR of *P. jadinii* M9 and *P. anomala* M10 were also evaluated. In both CChRs, the electron donors tested, NADH and NADPH alone had an specific activity (Fig. 6). A slight abiotic reduction of Cr(VI) by both, NADH and NADPH, was observed, but it was not substantial compared to Cr(VI) reduction with the CChRs plus either one of the electron donors. Subtracting NADH and NADPH unspecific effects, for *P. jadinii* CChR, specific activity was 17.5 nmol Cr(VI) min^{-1} mg $prot^{-1}$ and 13.5 nmol Cr(VI) min^{-1} mg $prot^{-1}$, respectively. *P. jadinii* CChR alone had an specific activity of 0.69 nmol Cr(VI) min^{-1} mg $prot^{-1}$, thus probing the necessity of an electron donor. In *P. anomala* CChR, discounting NADH and NADPH self-effects, chromate reductase activity was 19.2 nmol Cr(VI) min^{-1} mg $prot^{-1}$ and 18.98 nmol Cr(VI) min^{-1} mg $prot^{-1}$. CChR of *P. anomala* alone had an specific activity of 2.74 nmol Cr(VI) min^{-1} mg $prot^{-1}$. Cr(VI) reduction by the CChRs of *P. jadinii* M9 and *P. anomala* M10 was confirmed as a NAD(P)H-dependent reaction. Our work supports other studies reporting NADH or NADPH-dependent enzymatic reduction of Cr(VI) under aerobic conditions (Park et al., 2000; Mclean et al., 2000; Bae et al., 2005; Elangovan et al., 2006; Opperman et al., 2008). According to Ramirez-Díaz et al. (2008), the oxidation of NADH donates an electron to the chromate reductase enzyme and then, the electron is transferred to Cr(VI) reducing it to the intermediate form, Cr(V), which finally accepts two electrons from other organic substances to produce Cr(III).

Inhibition of the enzyme activity by selected metal ions was also determined (Fig. 7). In *P. jadinii* M9 CChR, only Cu^{2+} and Na^{+} produced an augmentation in the activity of 63 and 30%, respectively. All other ions tested had an inhibitory effect but in different levels. A decrease of 72% was observed with Hg^{2+}, while addition of Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{3+} and Zn^{2+}, resulted in a decrease of activity between 38 and 58%. In *P. anomala* M10 CChR, only Cu^{2+} produced a raise in activity of a 31%. Inhibition by Hg^{2+} and Mn^{2+} was higher than in *P. jadinii* M9 CChR, with a decrease in activity of 85 and 90%, respectively. Inhibition by Ca^{2+}, Mg^{2+} and Zn^{2+} was approximately 60%, while Fe^{2+} reduced the activity in a 32%. These results agree with those reported for *Arthrobacter crystallopoietes* (Camargo et al., 2004) and *Bacillus* sp. (Camargo et al., 2003; Elangovan

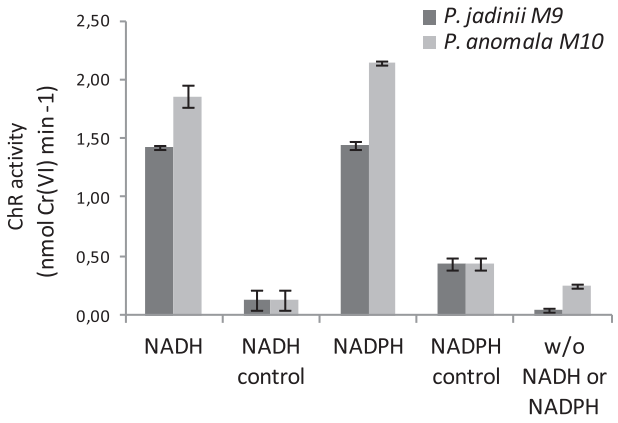


Fig. 6. Effect of electron donors NADH and NADPH in the Chromate reductase activity of CChRs from *Pichia jadinii* M9 and *Pichia anomala* M10. Data are mean of three independent replicates and error bars indicate standard deviation. Control: non-enzymatic reduction.

et al., 2006). Activation by Cu^{2+} can be explained considering that this transition metal may be acting as a prosthetic group for many reductases. Its main function would be related to the protection of electron transport, whether acting as a redox center or as an electron transporter between protein subunits. On the other hand, inhibition by Hg^{2+}, can be related with its affinity to −SH ligands, then suspecting the presence of this chemical group in the active site of the enzyme related to chromate reductase activity (Camargo et al., 2003).

3.1.4. Chromium initial concentration effect on reaction evolution

In *P. jadinii* M9 CChR, for initial concentrations of 0.4, 0.7 and 1 mM, a total removal of Cr(VI) was observed (Fig. 8(a)). This removal was obtained after 2 h for 0.4 and 0.7 mM, and after 5 h for 1 mM. An initial concentration of 1.3 mM, can be considered as inhibitory based on the detention of the metal removal at 60 min and the maintenance of Cr(VI) concentration at 0.83 mM. In *P. anomala* M10 CChR, initial concentrations of 0.4, 0.7, 1 and 1.3 mM a total removal of Cr(VI) was observed, at 2 h for 0.4 and 0.7 mM, at 3 h for 1 mM and at 4 h for 1.3 mM (Fig. 8(b)). A Cr(VI) initial concentration of 1.7 mM was reduced to 0.80 mM in 3 h and remained constant for the rest of the incubation time, suggesting that this one or a higher initial Cr(VI) concentration may be inhibitory. The effect of initial Cr(VI) concentration was also

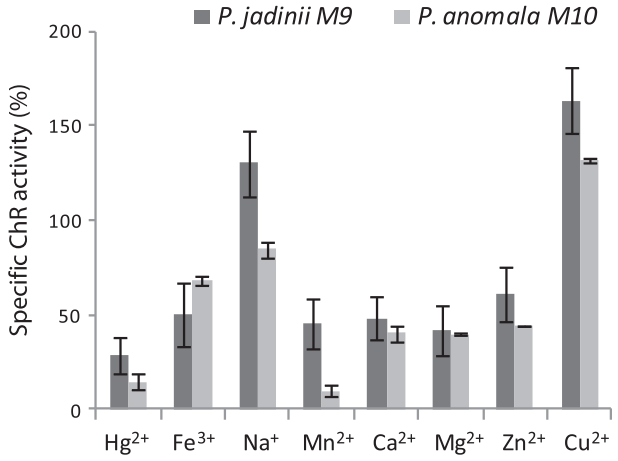
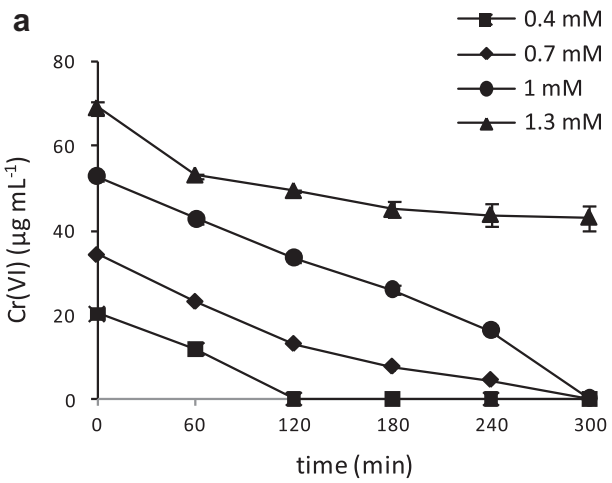


Fig. 7. Effect of metal ions in the Chromate reductase activity of CChRs from *Pichia jadinii* M9 and *Pichia anomala* M10. Data are mean of three independent replicates and error bars indicate standard deviation. Control without metal corresponds to 100%.



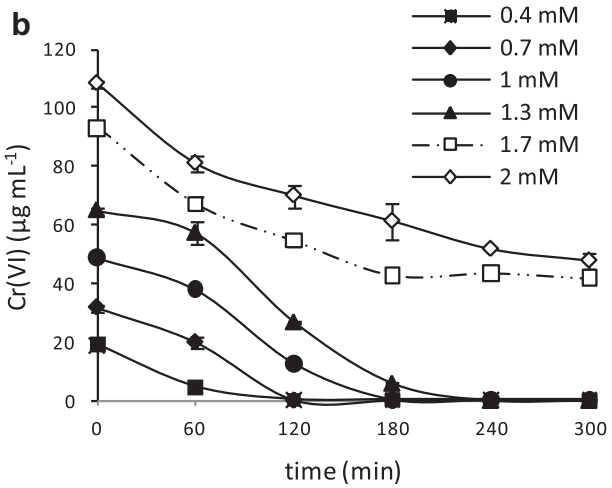


Fig. 8. Effect of initial Cr(VI) concentration in the Chromate reductase activity of CChRs from *Pichia jadinii* M9 (a) and *Pichia anomala* M10 (b). Data are mean of three independent assays and the error bars indicate standard deviation.

evaluated for *B. sphaericus* AND 303 in the range 0–500 μM, the authors observed a fast increment on the Cr(VI) reduction up to a concentration of 300 μM (Pal et al., 2005). Also, Sarangi and Krishnan (2007) reported lower chromium initial concentration values, between 75 and 192 μM. All these concentrations were in the μM order, showing the higher resistance of ChR activity in the CChR here in described with reference to the initial Cr(VI) concentration, which ranged between 400–2000 μM.

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